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BIOGENIC AMINES AND ACTIVE POLYPEPTIDES IN THE SKIN OF *LEPTODACTYLUS VILARSI* MELIN

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Abstract—*Leptodactylus vilarsi*, a rare Ecuadorian amphibian belonging to the "pachypus" section of the Leptodactylus genus, contains in its skin a set of biogenic amines. Bufotenidine, the ammonium quaternary base produced by *N*-methylation of 5-hydroxytryptamine is the predominant constituent of this set. Dehydrobufotenine, leptodactyline and histamine are also present, but *N*-methylated derivatives of histamine are lacking. Active polypeptides are represented by large amounts of a caerulein-like peptide. The glandular formations of the skin contain twenty to fifty times more active constituents than the remaining non-glandular skin. Taxonomically, *Leptodactylus vilarsi* appears to be related to *Leptodactylus pentadactylus dengleri* more closely than to any other Leptodactylus species of the "pachypus" section.

THE SKIN of the leptodactylid frogs of Central and South America belonging to the "pachypus" group of the genus *Leptodactylus* contains large amounts of aromatic amines and of caerulein-like polypeptides.[1–3] However, a considerable gap in the systematic screening of the leptodactylid frogs is represented by *Leptodactylus vilarsi*, a rare amphibian of Ecuador.

MATERIALS AND METHODS

Amphibian material. A large specimen of *Leptodactylus vilarsi* was captured in Ecuador in February 1968 and sent alive to Mendoza. The skin was removed from the animal immediately after its sacrifice and allowed to dry in the shade. It weighed 10·1 g. As shown in Fig. 1 the skin can be divided into two parts: the first constituted by diffuse glandular formations (1·5 g) and the second poor in cutaneous glands (8·6 g). They were separated, minced with scissors and then extracted for a week with 15 parts (w/v) of 80% methanol. The liquids were decanted and the skin fragments re-extracted for another week with 15 parts of the same solvent. The first extracts were combined with the corresponding second extracts and filtered.

Chromatography on alumina column. A satisfactory separation of the amines and polypeptides occurring in the methanol extracts of *L. vilarsi* skin was obtained by their chromatography on an alkaline alumina column. Alkaline alumina was a chromatographic grade product (activity I) obtained from Merck A.G., Darmstadt.

The extract of 0·5 g glandular skin was mixed with the extract of 2·83 g non-glandular skin (= 3·33 g total skin) and the liquid was evaporated nearly to dryness under

reduced pressure. The residue was taken up by stirring in 40 ml of 99% ethanol and the liquid was passed through a column of 40 g of alumina. Elution was performed at room temperature by successive addition of 33–66 ml each of 99, 95, 90, 80, 70, 60, 50, 30% ethanol, and 33 ml of distilled water. Fractions of 33 ml were collected.

Paper chromatography. Aliquots of crude methanol extracts or of eluates from the alumina column were suitably concentrated and then submitted to ascending paper chromatography using the solvent mixtures and the developing reagents described in

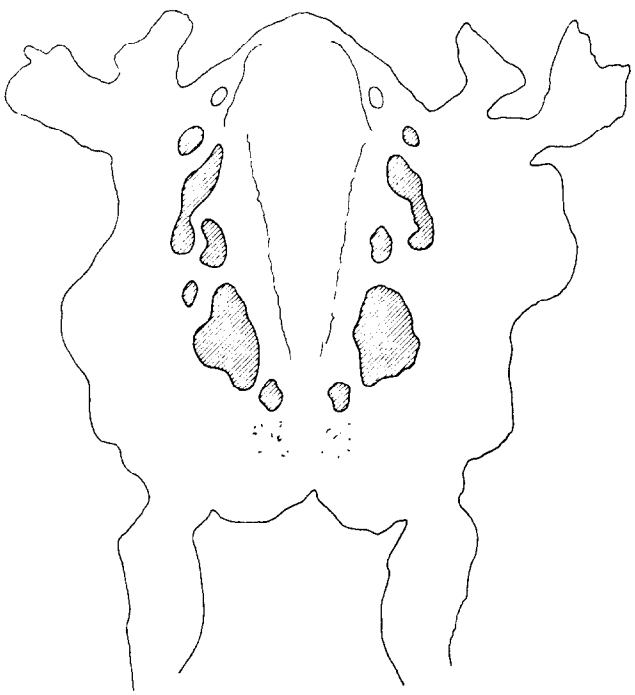

FIG. 1. Upper aspect of a dried skin of *Leptodactylus vilarsi*. Hatched areas represent the diffuse glandular formations.

detail in other papers.[1, 3] Semiquantitative estimation of the different indole- and imidazolealkylamines was carried out by visual comparison of the individual spots produced on paper chromatograms by different amounts of crude skin extracts or ethanol eluates with the spots produced by known amounts of the corresponding pure synthetic compounds.

Bioassay. Crude methanol extracts or eluates from the alumina column were deprived of the solvent in a boiling water bath under an air stream and the remaining aqueous liquid was brought to the desired volume with physiological saline. The liquids were then assayed on the isolated guinea-pig ileum, the isolated rat uterus, the blood pressure of the dog anaesthetized with sodium pentobarbitone (30 mg/kg, i.v.), and the *in situ* gall bladder of the anaesthetized guinea-pig.[4] Mepyramine was used as a histamine antagonist, and BOL(2-bromolysergic acid diethylamide) as a 5-HT antagonist.

Standard compounds. The following synthetic compounds were used for comparison: 5-hydroxytryptamine creatinine sulphate(5-HT, 0·43), *N*-methyl-5-HT creatinine sulphate (0·46), bufotenine base, bufotenidine iodide (0·66), dehydrobufotenine base, tryptophan; tyramine hydrochloride (0·74), candicine iodide (0·64), leptodactyline picrate (0·44); histamine dihydrochloride (0·6), imidazoleacetic acid, histidine, natural caerulein. This was prepared at the Farmitalia Laboratories for Basic Research, Milan. The amount of free bases in a gram of salt is given in parentheses.

RESULTS AND DISCUSSION

Results of paper chromatographic screening and of bioassay are summarized in Table 1 and Fig. 2. Data concerning *Leptodactylus pentadactylus dengleri* (Costa Rica, August 1964) which have been published in a preceding paper[1] were included for comparison.

TABLE 1. THE CONTENT OF BIOGENIC AMINES AND POLYPEPTIDES IN THE SKIN OF *LEPTODACTYLUS VILARSI* AND *LEPTODACTYLUS PENTADACTYLUS DENGLERI*

	Leptodactylus vilarsi (in μg free bases or caerulein per g dry tissue)			*Leptodactylus pentadactylus dengleri* (in μg free bases or caerulein per g dry tissue)
	Glandular skin	Non-glandular skin	Total skin	Total skin
5-Hydroxytryptamine	1600	65	300	20
N-Methyl-5-HT	65	?	10	1
Bufotenine	n.d.	n.d.	n.d.	n.d.
Bufotenidine	7900	310	1450	550
Dehydrobufotenine	200	?	35	3
Histamine	870	30	160	25
Leptodactyline	300	15	60	11
Caerulein-like peptide	4800	95	800	40

n.d., not detectable (< 1μg/g).

It appears from the tabulated data that the skin of *Leptodactylus vilarsi* presents the following spectrum of aromatic compounds and caerulein-like peptides:

Indole derivatives: 5-HT,*N*-methyl-5-HT,bufotenine,bufotenidine,dehydrobufotenine, tryptophan. The content of bufotenidine is particularly high.

Imidazole derivatives: histamine, histidine. It is possible that spot c is made up by imidazoleacetic acid.

Hydroxyphenilic derivatives: leptodactyline, spot a, spot b. On paper chromatograms spot a gives a wine-red colour with the Pauly reagent, spot b a red colour. They could not be identified with either tyramine or candicine.

Polypeptides: a caerulein-like polypeptide, appearing as an orange spot on paper chromatograms developed with the NNCD reagent. The caerulein-like peptide was identified and estimated chiefly by bioassay. Results obtained on the *in situ* guinea-pig gall bladder were in full accordance with results obtained on other test systems for caerulein.[4, 5]

The blood pressure response of the dog to i.v. injections of crude methanol extracts of *L. vilarsi* skin was the algebraic sum of the hypotensive effect of caerulein and the

hypertensive effect of bufotenidine. With low doses only the hypotensive effect was evident, with high doses the response was triphasic: abrupt, fleeting pressure fall followed by short-lived pressure rise, followed in turn by long-lasting pressure fall (Fig. 3).

The glandular skin contained twenty to fifty times more active constituents, per g tissue, than the non-glandular skin, demonstrating that both amines and peptides are produced and stored within the cutaneous glands and are presumably destined,

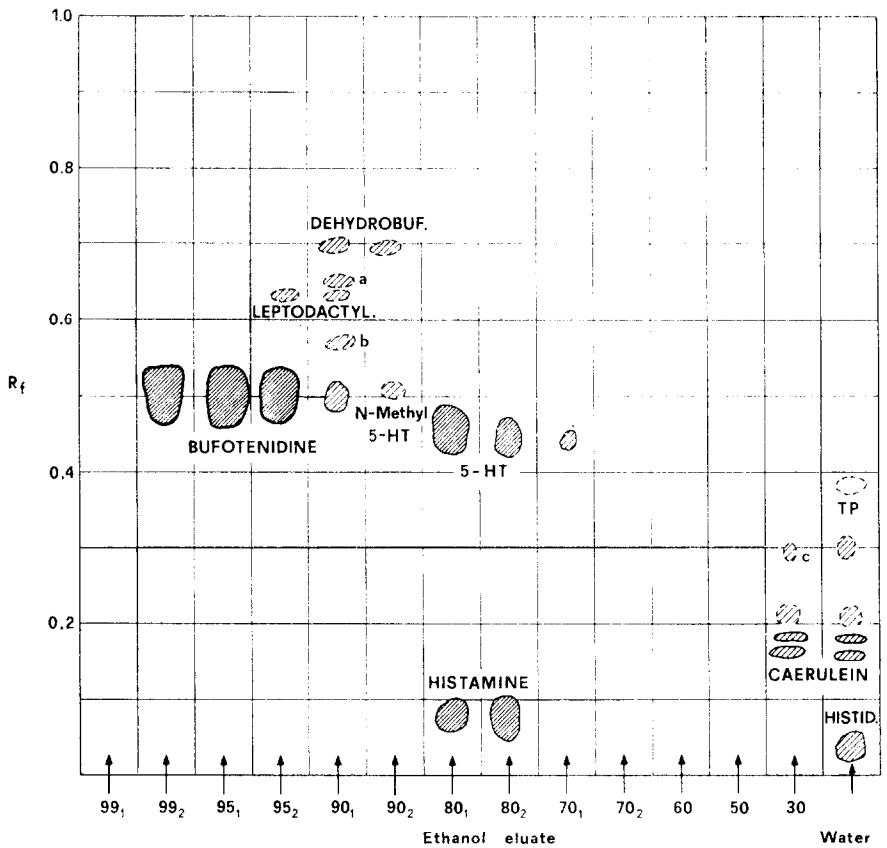

FIG. 2. Paper chromatograms of the ethanol eluates obtained from an alumina column loaded with an extract of the total skin of *Leptodactylus vilarsi*. Solvent, *n*-butanol–acetic acid–water; developing reagents, NNCD reagent and Pauly reagent. Amounts of eluates corresponding to 0·15 g of dry skin were applied on paper at arrows.

exactly as the other constituents of the so-called "venom", for external secretion. This should not be overlooked when trying to explain the biological significance of amines and peptides in the amphibian skin.

The content of caerulein in the glandular skin of *L. vilarsi* was the highest so far traced in a tissue. In this respect it should be remembered that the threshold i.v. dose of caerulein capable of stimulating the secretion of the dog pancreas and the motility of the dog and man gall bladder and small intestine is of the order of 0·2–1 ng/kg/min.

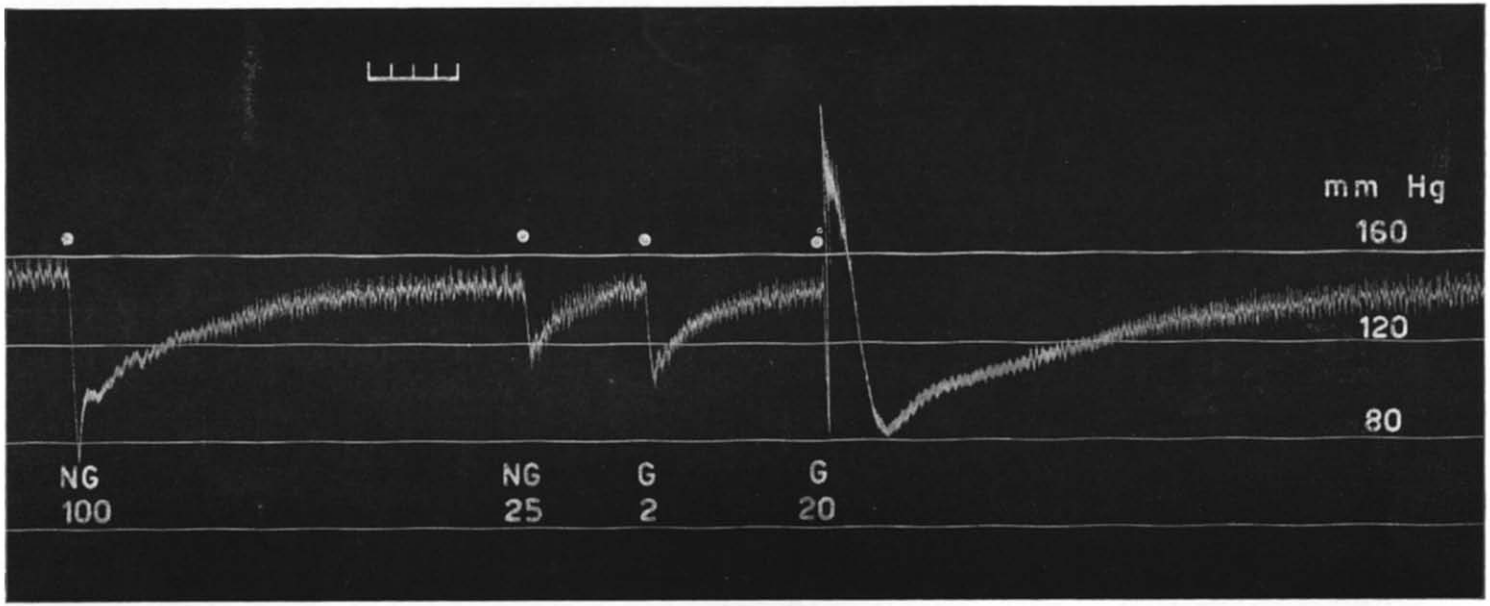

FIG. 3. Blood pressure of a dog weighing 15 kg, anaesthetized with sodium pentobarbitone (30 mg/kg i.v.). Time, 1 min. The effect of different doses, in mg of dry skin, of extracts of glandular (G) and non-glandular (NG) skin. On blood pressure glandular skin was approximately fifteen times as potent as non-glandular skin.

The spectrum of biogenic amines and peptides found in the skin of *Leptodactylus vilarsi* was very similar to that found in the skin of *Leptodactylus pentadactylus dengleri* and different from that found in other *Leptodactylus* species of the "pachypus" section. For example, *Leptodactylus pentadactylus pentadactylus* (Amazonia) lacked completely methylated derivatives of 5-HT in its skin, and the same was true for *Leptodactylus laticeps* (Formosa, North Argentina). The skin of *Leptodactylus pentadactylus labyrinthicus* (Misiones, North Argentina), in its turn, not only lacked methylated derivatives of 5-HT, but contained large amounts of *N*-methylated histamines and of spinaceamines.[1]

As repeatedly stated,[6, 7] occurrence of a given amine or of a given aminoacid aggregate implies the necessary activity of a set of enzymes, i.e. of specialized proteins built from regular subunits under the control of the genetic code.

Thus, on the basis of present data *Leptodactylus vilarsi* appears to be, from a taxonomical point of view, more closely related to *Leptodactylus pentadactylus dengleri* than to any other *Leptodactylus* species of the "pachypus" section.

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REFERENCES

1. V. Erspamer, M. Roseghini and J. M. Cei, *Biochem. Pharmac.* **13**, 1083 (1964).
2. A. Anastasi, G. Bertaccini, J. M. Cei, G. de Caro, V. Erspamer, M. Impicciatore and M. Roseghini, *Br. J. Pharmac.* (in press).
3. V. Erspamer, T. Vitali, M. Roseghini and J. M. Cei, *Biochem. Pharmac.* **16**, 1149 (1967).
4. G. Bertaccini, G. de Caro, R. Endean, V. Erspamer and M. Impicciatore, *Br. J. Pharmac.* **34**, 291 (1968).
5. G. Bertaccini, G. de Caro, R. Endean, V. Erspamer and M. Impicciatore, *Br. J. Pharmac.* **37**, 185 (1969).
6. J. M. Cei, V. Erspamer and M. Roseghini, *Systematic Zool.* **16**, 328 (1967).
7. J. M. Cei, V. Erspamer and M. Roseghini, *Systematic Zool.* **17**, 232 (1968).